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Hydrogen–Deuterium Exchange of a Primary Amide as a Model for Asparagine and Glutamine Exchange in Proteins[1,2]

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Hydrogen–deuterium exchange of the primary amide, isobutyramide, was investigated as a model for asparagine and glutamine–NH_2 exchange in a protein. A simple amide was chosen since the structures of several well-characterized proteins show most of these residues to be exposed to solvent. Isobutyramide-exchange data were obtained in 1:1 D_2O:dioxane solutions using a near-infrared method. The rate data were strictly pseudo-first order and yielded an average of 95% exchange of the primary amide hydrogens. In analogy with secondary amides, the pD dependence of the rate constants was characteristic of specific acid and base catalysis. In addition, analysis of the rate–pD profile for isobutyramide indicated a significant uncatalyzed exchange reaction. Temperature-dependence studies of the first-order rate constants at a fixed pD yielded an apparent activation energy of 19.3 kcal/mole. Predicted half-life times for the exposed primary amide hydrogens in proteins, based on these exchange parameters, indicate that asparagine and glutamine side chains generally would contribute to the overall rate data only below 15°C and then only for approximately 1 pD unit around the point of minimum reaction velocity.

Hydrogen exchange affords a sensitive method to detect conformational changes in biopolymers (*1–4*). However, during interpretation of protein exchange data questions often arise as to the source of the labile hydrogens. Usually the data of Eigen (*5*), Luz et al. (*6, 7*), and Grundwald et al. (*8*) on simple hydroxyl, carboxyl, and amine compounds are used to predict that the corresponding side-chain hydrogens of a protein will exchange too fast to measure on the normal time scale for macromolecular studies. This assumption was confirmed recently for lysozyme, since only amide hydrogen exchange could be detected after 5–10 min at 25°C (*9*).

Amide hydrogen exchange, on the other hand, is much slower ($t_{\frac{1}{2}} > 1$ min) at the pH of minimum exchange rate (*10, 11*). Although the fundamental region infrared method is specific for secondary amides, it does not generally afford any information regarding primary amide exchange. Furthermore, neither the near-infrared (*9*), nor the radio tracer technique, distinguishes primary from secondary (peptide) exchange in proteins. In addition, from one-fourth to one-half of the potential carboxylic side chains in several well-characterized proteins are amidated (Table 1). Consequently, interpretation of the

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rates and numbers of exchanging hydrogens from near-infrared, as well as radioactive tracer techniques, would be facilitated by information regarding the exchange behavior of a primary amide group.

TABLE 1

EXPOSED VERSUS BURIED PRIMARY AMIDE GROUPS IN PROTEINS OF KNOWN STRUCTURE

Protein	Total amino acids	Primary amides		Total asx + glx[a]
		Buried	Exposed	
Pancreatic trypsin inhibitor (*13*)	58	1	3	8
Horse cytochrome *c* (*12*)	104	2	6	20
Carp muscle myogen[b] (component 3) (*14*)	107	1	5	25
Hen egg white lysozyme (*15*)	129	1	15	26
Porcine pancreatic tosyl-elastase (*16*)	240	1	32	43

[a] The symbols "asx" and "glx" are used here to signify both the carboxyl and amide forms of the aspartic and glutamic acid residues.

[b] Some of the asn or gln residues in the carp myogen have been identified through analogies with those found for the corresponding hake protein.

Before choosing a suitable model for primary amide exchange, however, the environment of asn and gln residues in a protein needs to be established. Amides are considered ambivalent groups (*12*) and as such they could exist either on the surface or buried in the interior of a globular protein. A survey of several proteins of a known structure (Table 1), however, shows almost all of the asn and gln residues to be on the surface. Therefore, a simple primary amide should offer a reasonable model for these solvated side chains.

Apparently only one previous report (*17*) of primary amide exchange has been made. In that study the side-chain amide exchange of poly-DL-asparagine was separated from backbone peptide hydrogen exchange by kinetic analysis and the measurements were performed only at 0°C. Therefore, the present investigation was undertaken to determine the rates and temperature dependence of the primary amide hydrogen exchange in a kinetically simpler system.

MATERIALS AND METHODS

Isobutyramide, mp 127–129°C (Eastman); deuterium oxide (99.8 mole % D; Bio-Rad); NaOD and DCl in D_2O (Merck, Sharpe and Dohme); and reagent grade sodium phosphate (Baker and Adamson) were used as received. The *p*-dioxane (Matheson, Coleman, and Bell) was distilled over sodium before use.

Exchange measurements were performed using a Cary 14R spectrophotometer equipped with an 0–0.1 slide wire. Temperature regulation in the cell compartment utilized a Haake Model FE constant-temperature circulating bath combined with a Model R-30 refrigerated cooling unit.

Figure 1 shows a spectrum of IBA in 1:1 D_2O:dioxane recorded soon after mixing. The broad band centered near 1.5 μm is primarily due to the first overtone of the N–H stretching vibration. It appears to be a partially resolved doublet resulting from symmetric and asymmetric $-NH_2$ vibrations. The band at 1.40 μm is due to HOD and no doubt represents the first overtone of the OH stretching frequency. Consequently, the rate of exchange of the amide hydrogens with solvent deuterium can be followed in either the 1.5- or 1.4-μm regions.

Rate measurements in 1:1 D_2O:dioxane (v/v) were conducted similarly to those reported by Klotz and Frank (*11*). Solutions of 0.55 *M* isobutyramide (IBA) in dioxane and 0.02 *M* sodium phosphate in D_2O were preequilibrated separately to a few degrees

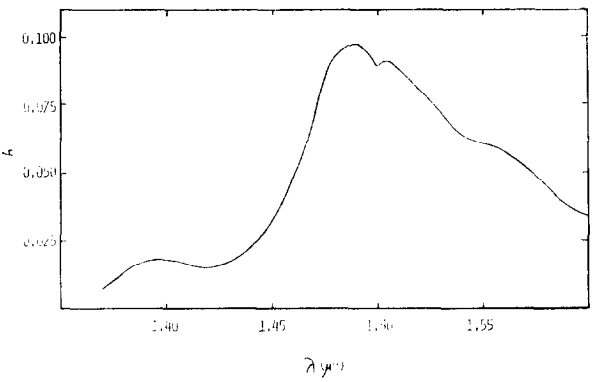

FIG. 1. Near-infrared spectrum of 0.275 *M* isobutyramide in 1:1 D_2O:dioxane containing 0.01 *M* phosphate buffer, pD 6.85, which was recorded 2 min after mixing using 2-cm cells at 15°C.

below the desired temperature. The pH of the buffer solution had been adjusted as needed with NaOD or DCl solutions in D_2O using a Beckman Century SS meter equipped with a combination electrode. After attaining proper temperature the reaction was commenced by mixing the two solutions, placing them in a 2-cm spectrophotometer cell, and sealing. The solutions were generally within ±0.5°C of the desired temperature when placed in the cell compartment and were within ±0.2°C in a few minutes. The moment of combination was defined as time zero (t_0) and absorbance readings were recorded continuously at 1.41 μm with occasional tracings at 1.24 μm which served as an internal standard. The increase in HOD with time, A_{HOD}, was defined as the absorbance at 1.41 μm minus that at 1.24 μm. Measurements were recorded from approximately 2 min to 1 hr or more after mixing and a complete exchange reading, A_∞, obtained after eight or more half-life times.

A molar extinction coefficient for HOD in 1:1 D_2O:dioxane at 15°C was determined for the 1.41-μm band. Small quantities of H_2O were added to a weighed quantity of D_2O solution in a spectrophotometer cell using a microburet equipped with a 0.5 μl/div. syringe attached to a small-bore Teflon tube. Spectra were recorded after the solution attained 15°C in the cell compartment. HOD absorbance was defined again as $A_{1.41}-A_{1.24}$ to make ε_{HOD} compatible with the exchange measurements.[3]

[3] Actually, HOD absorbance at 1.24 μm is almost insignificant.

Temperature dependence of the exchange rate was determined at 7.9, 10.0, 12.9, 15.0, 17.8, and 19.9°C and for a pD (=$pH_{\text{meter reading}} + 0.4$) of 6.4. The pD measurements were rechecked after A_∞ had been determined and found not to vary more than ±0.03 units. The pD readings were taken at room temperature, since it had been found that the pH of a 0.01 *M* deuterio–phosphate buffer in 1:1 D_2O:dioxane solution increased by only 0.04 units upon cooling from 25 to 10°C. That difference was obtained by measuring the buffer solution at 25°C, when the meter had been standardized at 25°C, relative to the buffer at 10°C when the meter had been standardized at 10°C.

Calculations and Results

Plots of $\log(A_\infty - A_{\text{HOD}})$ vs t for a given pD were prepared and the exchange rates were found to be strictly first order over several half-life times as illustrated in Fig. 2. Typically, linear regression correlation coefficients of 0.999, or greater, were obtained.

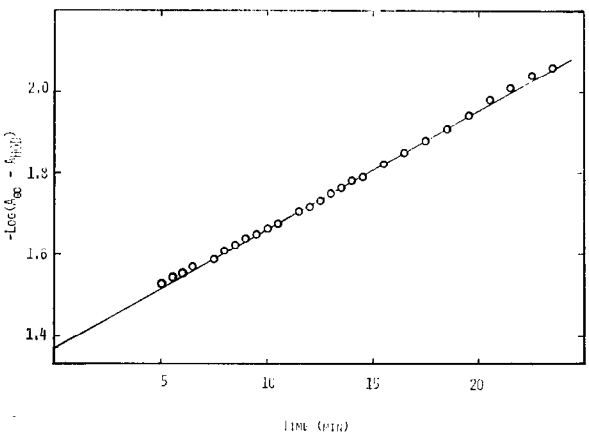

FIG. 2. First-order plot of the increase in HOD absorbance at 1.41 μm for isobutyramide in 1:1 D_2O:dioxane in 0.01 *M* phosphate buffer, pD 6.54, 15°C.

Furthermore, the intercept of the $\log(A_\infty - A_{\text{HOD}})$ vs t plot gives $\log \Delta A$, the change in absorbance at 1.41 μm between t_0 and complete exchange. Using these ΔA values, percentage exchange calculations for each run were obtained from

$$\% \text{ exchange} = \frac{\Delta A}{\Delta A_T},$$

where ΔA_T is the theoretical absorbance change at 1.41 μm based on the concentration of IBA in the 2-cm cell and the measured ε_{HOD} of 0.041 M^{-1} cm^{-1}. The data illustrated in Fig. 3 gave percentage exchanges ranging from 81 to 103% with an average of 95%. Consequently, the first-order exchange kinetics accounts for virtually all the amide hydrogens of IBA.[4]

[4] Although efforts were made to keep the IBA sample dry, amides readily hydrate, which could account for the % exchange of less than 100%. Any absorbed water, however, would exchange instantaneously by the present technique and not contribute to the observed ΔA.

The pD dependence of the rate constants at 15°C (Fig. 3) shows a specific acid and base catalytic profile similar to those of secondary amides (*11*). The catalytic constants were calculated from Eqn. 1 as previously described (*18*),

$$k = k_0 + k_D(D^+) + k_{OD} K_{D_2O}/(D^+), \quad (1)$$

where k is the observed rate constant; k_0 an uncatalyzed exchange rate constant; k_D and k_{OD}, the specific acid and base catalytic constants, respectively; K_{D_2O}, the autoionization constant for D_2O in the solvent; and (D^+), the deuterium ion activity. Two

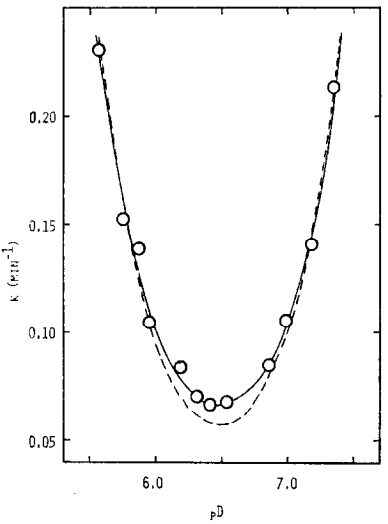

FIG. 3. Rate-pD profile for isobutyramide in 1:1 D_2O:dioxane. 15°C. The points are experimental and the dashed and solid lines were calculated from Eqs. 2 and 3, respectively.

methods were used to analyze the data. First a least-squares calculation with $k_0 = 0$ was performed and gave an overall 1σ error of 0.0089 in units of k. Secondly, calculations including a k_0 term were made which gave $\sigma = 0.0061$. The equations for the 2- and 3-parameter fits are

$$k = (8.7 \pm 0.2)10^4(D^+) + (9.4 \pm 0.3)10^{-9}/(D^+) \quad (2)$$

$$k = (0.014 \pm 0.002) + (7.9 \pm 0.2)10^4(D^+) + (8.5 \pm 0.3)10^{-9}/(D^+) \quad (3)$$

and the calculated curves are illustrated in Fig. 3 by the dashed and solid lines, respectively. The two curves are comparable at the extreme pD values on either side of the point of minimum exchange rate, pD_{min}, but the 2-parameter curve fails to account satisfactorily for the data in the vicinity of pD_{min}. That would be expected if there were a significant pD independent rate of exchange for IBA, since k_0 would then make a smaller percentage contribution to k at higher or lower pD values.

An Arrhenius plot of the rate constants determined at pD 6.40 ± 0.03 between 8 and 20°C is given in Fig. 4. Linear regression analysis gives a slope equivalent to $E^* = 19.3$ kcal/mole with a correlation coefficient of 0.994.

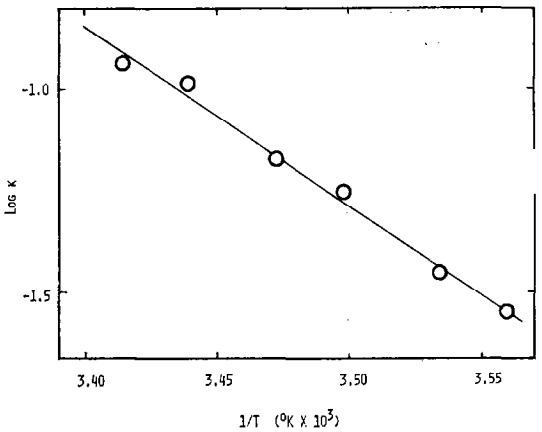

FIG. 4. Plot of $\log k$ vs $1/T$ for the hydrogen exchange of isobutyramide in 1:1 D_2O:dioxane at pD 6.40.

DISCUSSION

If pD_{min} is independent of temperature, which has been the case with almost all amides (*19–22*), it can be shown from Eq. (1) (*19*) that the apparent activation energy for the acid-catalyzed reaction, E_D^*, is numerically equivalent to that found on the basic side of pD_{min}, E_B^*.

$$E_D^* = E_B^* = E_{OD}^* + \Delta H_{D_2O}$$

However, E_B^* includes the change in enthalpy for ionization of deuterium oxide, ΔH_{D_2O}, as well as the apparent activation energy of specific base catalysis, E_{OD}^*. Under such circumstances, and on the basis of acid and base catalysis alone, it would not matter what pD is selected to measure E_D^*.

H–D exchange in IBA at 15°C, however, shows a significant k_0 term which will contribute to activation energies determined at pD 6.4. Although the Arrhenius plot of Fig. 4 is strictly linear, it can be shown using the exchange constants of Eq. (3) that the temperature range is not sufficient to detect any curvature resulting from two exchange processes of different activation energies. In particular, $E_D^* = 19.3$ and $E_0^* = 30$ kcal/mole will give a correlation coefficient of greater than 0.998 over the same temperature range, since the contribution for k_0 is only about 20% at pD 6.4. Consequently, the observed E^* represents an average value with a small contribution from E_0^*. However, k_{min} at other temperatures are predicted more accurately using E^* than E_D^*.

Table 2 gives the H–D exchange parameters for *N*-methylacetamide (NMA) in 1:1 D_2O:dioxane and in pure D_2O at 25°C along with observed and predicted values for IBA. Comparison of k_{min} for the two compounds at 25°C shows IBA to exchange faster by a factor of 2.7. One-way primary amide exchange could be faster is that the two hydrogens could afford a statistical advantage. However, the accepted mechanism for acid-catalyzed hydrogen exchange in amides (*24*) involves a slow protonation of the nitrogen followed by a rapid loss of a hydrogen. Similarly, the slow step for base catalysis is the abstraction of a hydrogen followed by rapid reaction with solvent. Consequently, if the mechanisms are correct, there should not be a statistical factor involved

in the acid-catalyzed reaction, but one would be anticipated for base exchange. Under these circumstances k_{min} would shift to a lower pD_{min} due to the accelerated base catalyzed reaction. However, pD_{min} for IBA is 0.28 units higher than that for NMA. Apparently, the positive inductive effects of the isobutyl moiety have increased the basicity of the amide group, thereby sufficiently enhancing the acid-catalyzed reaction to override any possible statistical factors.

TABLE 2

HYDROGEN–DEUTERIUM EXCHANGE PARAMETERS FOR PRIMARY AND SECONDARY AMIDES

Compound	Temp (°C)	k_{min}	pD_{min}	k_D (M^{-1} min^{-1})	$k_{OD}K_{D_2O}$[c] (M min^{-1})
1:1 D_2O:Dioxane					
Isobutyramide	15	0.066	6.48	7.9×10^4	8.5×10^{-9}
N-Methylacetamide (*19*)	25	0.078	6.20	4.0×10^4	2.1×10^{-8}
Isobutyramide[a]	25	0.21	6.48	—	—
D_2O					
N-Methylacetamide (*20*)	25	0.48	5.42	5.5×10^4	7.3×10^{-7}
N-Ethylisobutyramide (*21*)	25	0.18	5.70	—	—
Isobutyramide[b]	25	1.27	5.70	—	—

[a] Calculated value using $E_D^* = 19.3$ kcal/mole.

[b] Calculated values; see text for details.

[c] $K_{D_2O} = 5.65 \times 10^{-16}$ and 1.35×10^{-15} at 15 and 25°C, respectively (25) in the pure solvent but are reduced by a factor of about 265 in 50:50 dioxane: D_2O (26).

The data for NMA in D_2O (Table 2) show that k_D changes little in passing from 1:1 D_2O:dioxane to pure solvent but that $k_{OD}K_{D_2O}$ changes by a factor of 30. This results in a downward shift in pD_{min} by 0.78 units and an increase in k_{min} by a factor of six. Based on the accepted mechanism, any change in k_{OD} for a compound would be expected to show a corresponding change in k_D. Therefore, the observed change in $k_{OD}K_{D_2O}$ probably reflects a change in K_{D_2O} as first pointed out by Klotz and Frank (*11*).

The solvent effects observed for NMA can be used to predict the behavior of IBA in pure D_2O. Application of the appropriate factors to k_{min} and pD_{min} for IBA lead to the values listed in the last row of Table 2. Interestingly, the predicted pD_{min} for IBA in pure D_2O is identical to that observed for *N*-ethylisobutyramide in the same solvent. k_{min} for IBA, however, is about seven times larger than for the *N*-ethyl derivative which is greater than the enhancement seen between IBA and NMA. Furthermore, the *N*-ethylisobutyramide also shows an increase of 0.28 units in pD_{min} compared to that for NMA. Apparently, the carbonyl carbon substituent of the amide group primarily determines the position of pD_{min} while that of the nitrogen is the major rate-affecting factor.

Comparisons are also possible with the data of Molday and Englander (*17*), who used a Sephadex method to study hydrogen–tritium exchange of poly-DL-asparagine. At 0°C they reported a k_{min} of 0.083 min^{-1} and a pD_{min} of 4.8. Using the differences in exchange behavior for NMA in 1:1 D_2O:dioxane and pure D_2O as a basis, the predicted

values for IBA in D_2O at 0°C are 0.082 min^{-1} for k_{min} and 5.7 for pD_{min}. Although the rate constants agree fairly well, pD_{min} values do not. The lowered pD_{min} for the asparagine side chain may reflect inductive effects from the dipeptide backgone.

In terms of expected exchange behavior of primary amide side chains of proteins in D_2O, Table 3 lists $t_{\frac{1}{2}}$ at pD_{min} based on the behavior of IBA. These data can be used to estimate whether or not primary amide hydrogens in proteins will contribute significantly to observed exchange, since k_{min} for IBA at 0°C was the same as that for poly-DL-asparagine. However, the most appropriate value of pD_{min} for exposed asn and gln amide hydrogen exchange is no doubt the 4.8 observed for poly-DL-asparagine (17).

TABLE 3

PREDICTED EXCHANGE HALF-LIFE TIMES FOR PRIMARY AMIDE HYDROGENS OF EXPOSED ASPARAGINE AND GLUTAMINE RESIDUES AT pD_{min}

t (°C)	$t_{1/2}$ (min)
0°	8.4
15°	1.7
25°	0.5

Consider, for example, an exchange study of a protein containing 10 asn and gln residues, begun 5 min after the reaction is initiated, and using an analytical procedure precise to ±2 hydrogens. At 0°C only about 5 of the 20 primary amide hydrogens will have exchanged at pD_{min} when the first measurement is made. Consequently, asn and gln side-chain hydrogens will make a substantial contribution to the observed kinetics at pD_{min} and 0°C. To the contrary, at 25°C essentially all 20 hydrogens will have exchanged in 5 min at pD_{min}. The break point for the present conditions occurs near 15°C where approximately 2 primary amide hydrogens remain unchanged in 5 min. In addition, at pD values of only 0.5 units on either side of pD_{min} these side-chain hydrogens would contribute for even shorter intervals.

In general, exposed primary amide hydrogen exchange in a protein can be expected to contribute to rate data only over approximately a pD unit interval about pD_{min} and then only below 15°C.

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